# Utilization of Cytochrome $b_{562}$ as a Localized Labile Heme Chelator

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# Utilization of Cytochrome $b_{562}$ as a Localized Labile Heme Chelator

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## Abstract:

Heme is an essential, but toxic cofactor required for virtually all aerobic life. As a consequence, cells are challenged to safely traffic heme to hemoproteins that reside in every subcellular compartment. However, the mechanisms underlying heme transport and trafficking are largely unknown. Moreover, it is unclear how various subcellular compartments communicate their requirement for heme to the mitochondria, where heme is synthesized. In order to determine how different subcellular compartments sense and respond to heme deficiency, I have been developing a heme chelator to induce local heme deficiencies. Once this is achieved, we can employ transcriptome and proteome profiling to determine pathways that enable various organelles to adapt to heme deficiency. Altogether, we seek to better understand how cells appropriate and distribute heme to diverse compartments that require this essential nutrient.

## Introduction:

Heme is a prosthetic group that is present in many proteins in the human body. Hemeproteins can play a variety of roles in the cell, such as catalysis of certain metabolic reactions, movement of electrons within the cell, and inter/intracellular gas binding and movement.[1] Heme has been traditionally considered as a static cofactor that is buried within hemoproteins. However, recent results utilizing a genetically modified fluorescent hemoprotein as a heme sensor have shown that there is a pool of labile heme, a heme molecule not tightly bound to hemoproteins, that may act in various cellular functions such as signaling.[1] Although heme is a vital component to many cellular functions, free heme is cytotoxic to the cell due to its ability to oxidize oxygen to a radical species through Fenton reactions. Therefore, heme must be highly regulated within the cell.[2] Mis-regulation of both labile heme and hemeproteins has been linked to pathologies that include cancer and heart disease.[3,4] Understanding how labile heme regulation plays a role in these diseases may lead to new treatments.[1]

Currently, it is unknown which organelles "need" labile heme the most. If there was a localized cellular deficiency in the cell, which organelle would be first to lose its labile heme pool to recover from this deficiency? And just as interesting, which organelle would attempt to maintain its free heme pools the longest before donating it to the site of the deficiency. For example, the nucleus uses labile heme to activate nuclear transcription factors such as *hap1*.[5] Therefore, it could be hypothesized that the nucleus would be the last compartment to transfer

labile heme to a deficient area. The goal of this project was to develop a tool to test similar hypothesis.

To be capable to answer questions of such an unknown phenomenon, I have created a local heme deficiency using high affinity hemoprotein cytochrome $b_{562}$. Overexpression of this protein created a heme sink in any compartment in which the construct is overexpressed. Further testing of the effect of cytochrome $b_{562}$ on different cell lines was also accomplished.

## Literature Review:

Heme b plays a role in various intracellular biochemical pathways due its chemical structure.[6] The heme molecule consists of a protoporphyrin IX ring around a positively charged iron atom. Because the protoporphyrin IX ring is extremely hydrophobic, free heme can disrupt cell membranes and cell signaling. The positively charged iron atom in the center of this hydrophobic ring is known to create toxic free radicals within the cell. These free radicals can lead damage of cellular DNA and various other organelles. Due to this cytotoxicity, heme must be tightly regulated within the cell.[1] Because of this, scientists have previously considered heme to be kinetically inert and tightly buried within hemeproteins. Recent research has shown that there is another pool of "labile" heme that makes up 10% of the total heme pool.[7] Labile heme is available for heme signaling and various heme dependent processes such as hemelyation. This pool of labile heme must also be tightly trafficked and controlled in the cell, or else it could lead to various pathologies such as cancer.[8]

Currently, there is not much that is known about how this pool of labile heme is trafficked within eukaryotic cells to better understand this baker's yeast (Saccharomyces Cerevisiae), a model eukaryotic organism, will be used. This field of research is critical because many diseases such as cancer and heart disease have been previously linked to heme levels. Recent research has been performed to better understand the labile heme pool and its regulation. Heme trafficking factors such as heme importers/exporters (FLVCR1a and PUG1), heme

proteins (Nitric Oxide Synthase), and heme buffering factors (GAPDH). In addition, various small molecules such as nitric oxide and hydrogen peroxide have been shown to impact labile heme levels as well.[9]

Cytochrome $b_{562}$ had been previously cloned into a low copy number plasmid and expressed in order to sequester labile cytosolic heme. This sequestering has allowed for determination of cellular signaling processes that are dependent on the presence of labile heme. For example, the use of this cytosolic heme chelator has shown that regulation of nuclear transcription factor *hap1* could be dependent on more than levels of cellular heme synthesis. Sequestering labile heme may also lead into insights on various diseases that labile heme is misregulated in such as cancer or alzheimer's.[7]

A valuable tool in determination of these discoveries has been the creation of ratiometric heme sensors by Hanna et. al. These ratiometric sensors utilize hemeprotein Cytochrome $b_{562}$ that is fused to both mKATE and eGFP as fluorescent reporters. When heme binds to the Cytochrome $b_{562}$, eGFP fluorescence is quenched while mKATE fluorescence is constant. This tool allows for readouts of labile heme in the cell.5

The previous study utilized Cytochrome $b_{562}$ in a low copy number plasmid. I have inserted the Cytochrome $b_{562}$ into a high copy number plasmid. The purpose of this is to allow for higher numbers of the Cytochrome $b_{562}$ to be transcribed. This will allow for greater sequestering of cytosolic heme. In addition to this, subcellular localization tags will be added to the Cytochrome $b_{562}$ construct to allow for localized heme deficits. We can monitor these deficits by utilizing ratiometric heme sensors previously mentioned and various other techniques such as

microscopy and *hap1* activity. Observing this phenomenon will allow for a better of understanding of labile heme's role in the cell and how the cell responds to localized heme deficiencies.

## Materials and Methods:

To generate this tool, a primer set was generated to amplify the Cytochrome $b_{562}$ gene from the plasmid pRS 316 and a FLAG tag was introduced at the N-terminus of the Cytochrome $b_{562}$ sequence. The primer was generated to contain an *Spe*I restriction site at the 5' end of the forward primer, and a *Sac*I restriction site at the 3' end of the reverse primer. The sequence of the primer has been provided in the SI. A PCR was then ran according to the settings present in the SI. The DNA was then purified via DNA miniprep. The purified DNA was stored at 0° celsius until later use. The amplified Cytochrome $b_{562}$ was then digested using both *Spe*I and *Sac*I. The digested product was then ligated into plasmid pRS 426 GPD according to the ligation protocol in the SI. A test digest was then ran in order to verify the identity of the plasmid. The protocol and test digest results can be found in the SI.

Another set of primers was generated to amplify the Galactose promoter from pRS 316 GAL. The forward primer was designed to introduce a EcoRI restriction site at the 5' end of the gene. The reverse primer was designed to introduce a BamHI site at the 3' end of the gene. The PCR reaction was then ran to amplify this fragment from the original plasmid. The amplified Gal promoter sequence was then digested using *Eco*RI and *Bam*HI. A ligation reaction was then performed to insert the digested product into the pRS 426GPD+Flag-Cyt $b_{562}$ construct. A test digest was then ran to ensure the correct construct was generated.

Once the plasmid was generated, it was transformed into wild type and *hem1Δ* yeast cells. Next, an anti-flag western blot was then performed to ensure the galactose titratable system was operational. Yeast were grown to 1 OD in in SC-URA-LEU media that either contained 1% or 0% galactose. The samples were then lysed, and the lysate was ran on an 10% Acrylamide gel. The gel was then transferred to a western blot membrane overnight. The membrane was then stained with rabbit anti-FLAG antibody.

Next, cytosolic labile heme sensors HS1 and HS1M7A were cloned into both the wildtype and the *hem1Δ* yeast strains. To determine whether, the sensors are being expressed, plate reader fluorescence was taken at two settings. The first setting was an excitation wavelength of 488 nm and an emission wavelength of 510 nm, this was to confirm the fluorescence of eGFP. Another reading was taken at 588 nm excitation and a 635 emission. This was done to determine that there was mKATE fluorescence.

After confirmation that overexpression only occured at 1% galactose concentrations and the proper labile heme sensors were present in each strain. Both wildtype and *hem1Δ* strains either with or without the Flag-Cyt $b_{562}$ were grown in SCE-LEU-URA media to a OD of 1. The fluorescence ratios of each sample was then taken to determine relative labile heme levels.

## Results:

Generation of a plasmid that contained the Cytochrome $b_{562}$-FLAG construct under control of a galactose promoter system was confirmed through the western blot shown in figure 1. No band in the 0% lane is indicative of lack of FLAG tag in the sample. There is a clear dark band in both yeast strains at 1% galactose, indicative of transcription of the FLAG construct.

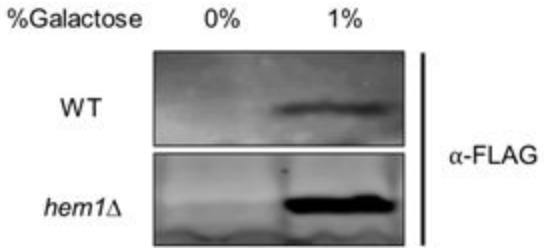


***Figure 1:*** *Cytochrome $b_{562}$ expression under galactose induction. Cyt $b_{562}$ expression was induced with 1% galactose in the media. The expression of Cyt $b_{562}$ was monitored via immunoblotting using an anti-FLAG tag antibody. The darker the band, the more copies of protein are present.*

When the sensor was expressed in wild type yeast, there was a statistically significant decrease in labile heme levels after both t-tests and post hoc tests were performed, this is shown in Figure 2. However, in *hem1Δ* yeast, there was no statistically significant difference between the induced and uninduced samples. An enlarged image of the wildtype sample is shown in figure 3. Induced wildtype samples had two times less relative labile heme levels compared to un-induced wild type samples.

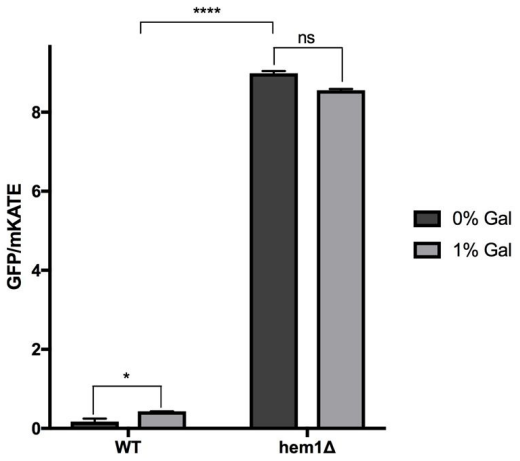


**Figure 2:** Wildtype and *hem1Δ* yeast containing Cytochrome $b_{562}$ Galactose induced vs uninduced. Labile heme levels were measured using HS1 sensor in triplicate. Both t-tests and and post hoc tests were performed to determine statistical relevance.

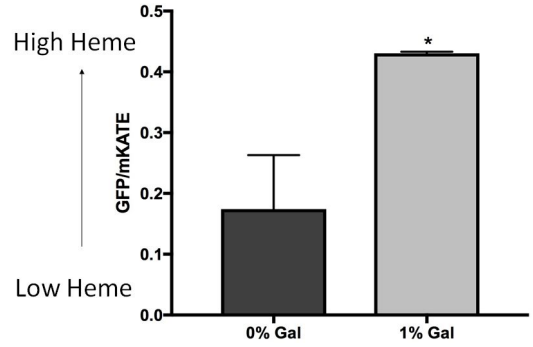


**Figure 3:** Wildtype Yeast containing the Cytochrome $b_{562}$ Galactose induced vs uninduced. Labile heme levels were measured using the HS1 sensor in triplicate.

## Discussion:

This data shows that the chelation of labile heme through over expression of Cytochrome $b_{562}$ in Wildtype yeast was successful. This is proved through the 1% galactose sample containing a higher ratio of eGFP/mKATE, indicating lack of labile heme. These results are further validated through the usage of both an ANOVA and a t-test. Although there was a 2 fold increase in fluorescence in the induced sample, it would be optimal if there was a greater chelation of labile heme. This gives further motivation for future studies with the goal of fine tuning the utilization of this tool in wildtype yeast.

Unlike wildtype cells, *hem1Δ* cells did not have any noticeable chelation of heme with overexpression of Cytochrome $b_{562}$. There are some some possible explanations for this phenomena. One said explanation is that the labile heme levels in *hem1Δ* are too low for a sensor such as HS1. This could lead to differences in labile heme being below the limit of detection for this specific sensor. To determine whether this is the case, a sensor with a different affinity for labile heme, such as HS1M7A, could be cloned into *hem1Δ* already containing the construct. Another hypothesis is that the *hem1Δ* cells may be unable to support the expression of both a labile heme sensor and the Cytochrome $b_{562}$ construct at the same time due to the cells being unable to reach the metabolic requirements.

Moving forward, there are multiple potential paths to explore to better both the expression and the effectiveness of this construct. One reason that this construct is not as effective as expected could be due to the disruption of function, structure, or both, by the FLAG tag. To remedy this, different polypeptide tags, such as a poly-histidine tag, can be tried. In addition, it may be possible for greater expression of the construct if it was cloned into a

different plasmid utilizing a different expression system, such as a phosphate or copper dependent system. Along with previously mentioned tactics, it may be possible to more effectively chelate labile heme with Cytochrome $b_{562}$.

After optimization of this tool, there are many potential applications and uses. One of these applications is attaching different sub- compartimental localizations tags to Cytochrome $b_{562}$ to shuttle the protein into different compartments of the cell such as the nucleus, or mitochondria. This will allow for the creation of subcompartment specific deficiencies, which may aid to further discoveries in the field of heme trafficking, when coupled with other investigative methods such as proteomic techniques.

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